Fate of Donor Centrosome and Microtubule Dynamics of Porcine Somatic Cell Nuclear Transfer Embryos

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ABSTRACT

We investigated the microtubule dynamics, including the inheritance of donor centrosomes and the mitotic spindle assembly occurring during the first mitosis of somatic cell nuclear transfer (SCNT) embryos in pigs. SCNT embryos were fixed 15 min and 1 h after fusion in order to assess the inheritance pattern of the donor centrosome. The distribution and dynamic of the centrosome and microtubule during the first mitotic phase of SCNT embryos were also evaluated. The frequency of embryos evidencing γ-tubulin spots (centrosome) was 93.2% in the SCNT embryos 15 min after fusion. In the majority of the SCNT embryos (61.5%), however, no centrosome was observed 1 h after fusion. The frequency of the embryos with no or abnormal mitotic spindles 20 h after fusion was 19.6%. The γ-tubulin spots were detected near the nuclei of somatic cells regardless of cell cycle phase, whereas γ-tubulin spots in the SCNT embryos were observed only during the inter-anaphase transition. These results showed that the donor centrosome is inherited into the SCNT embryos, but failed to assemble the normal mitotic spindles during first mitotic phase in some SCNT embryos.

(Key words : Microtubule, Centrosome, γ-Tubulin spots, Porcine SCNT)

INTRODUCTION

In mammalian embryos, the spindle microtubule performs a central function in genetic inheritance, to ensure the normal development of each blastomere. The microtubule is formed from tubulins, heterodimers composed of α-and β-tubulins, and is organized by large proteinaceous structures, which are termed microtubule organizing centers (MTOC). The principal MTOC in animal cells is the centrosome, which consists of a pair of perpendicularly arranged centriols surrounded by pericentriolar material (PCM). The PCM is a protein mass that plays a role in microtubule nucleation (Gould and Borisy, 1977), and an abundant quantity of γ-tubulin is located in the pericentriolar region of mammalian centrosomes (Stearns *et al.*, 1991). The γ-tubulin is probably the functional unit of MTOC that nucleates and stabilizes the centriolar microtubules (Raff *et al.*, 1993; Moritz *et al.*, 1995; Zheng *et al.*, 1995; Moudjou *et al.*, 1996).

During the mitosis of somatic cells, a nucleus condenses into a pair of chromosomes and segregates to opposite poles by the mitotic spindle. Simultaneously, centrosomes also segregate and migrate around the nucleus until two sets of microtubule arrays establish the bipolar spindle. The centrosomes are not observed in the mature oocytes of the pig (Kim *et al.*, 1996), sheep (Le Guen and Crozet, 1989) and cow (Long *et al.*, 1993), but functional MTOCs are present at spindle poles that do not harbor centrioles. In the zygotes of these species, the sperm centriole organizes a fully functional MTOC by the recruitment of dispersed centrosomal proteins in the oocyte cytoplasm (Schatten, 1994; Stearns and Kirschner, 1994). In nuclear transfer, a recipient oocyte is enucleated by the removal of the MII nucleus and surrounding materials, including the meiotic spindle apparatus. Afterward, a new nucleus and centrosome from the donor cell should be introduced into the recipient cytoplasm.

We examined the microtubule dynamics, most notably the inheritance of donor centrosomes following nuclear transfer and the mitotic spindle assembly including its morphological changes during the first mitosis of SCNT embryos in pigs.

MATERIALS AND METHODS

In Vitro Maturation

Porcine ovaries were obtained from a local slaughterhouse. Cumulus-oocyte complexes (COCs) were collected by aspiration from ovary antral follicles (3~6

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mm diameter). The follicular fluid was pooled into 50 ml conical tubes and the sediment was washed in Tyrode's lactate (TL)-Hepes containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma, St. Louis, MO, USA). For the maturation culture, COCs were cultured in NCSU-23 medium (Petters and Wells, 1993) supplemented with 0.6 mM cysteine (Sigma), 10 ng/ml of epidermal growth factor (EGF; Sigma), 10 IU/ml of pregnant mare's serum gonadotropin (PMSG; Intervet International B.V., Boxmeer, Holand), and 10 IU/ml of human chorionic gonadotropin (hCG; Intervet International B.V.), 50 μg/ml of gentamicin (Sigma) and 10% (v/v) porcine follicular fluid for 22 h at 39℃ in an atmosphere of 5% CO_2 in air. COCs were then cultured for 20 h under identical conditions and medium without hormone.

Micromanipulation

After maturation, the cumulus cells were removed by the vortexing of the COCs in PBS (Gibco-BRL, Grand Island, NY, USA) containing 0.1% PVA and 0.1 % hyaluronidase (Sigma). The oocytes were enucleated in Hepes-buffered TCM-199 (Gibco-BRL) supplemented with 3 mg/ml of BSA and 5 μg/ml of cytochalasin B (Sigma) by aspiration of the first polar body and MII plate in a small quantity of surrounding cytoplasm. Enucleation was verified by staining the oocytes with 1 μg/ml of Hoechst 33342 for 15 min at 39℃. Donor cells were obtained from a Day 50 crossbred female porcine fetus. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 50 μg/ml of gentamicin for 2~3 days. Prior to NT, the cells were cultured for an additional 5 days in DMEM supplemented with 0.5% FBS. A single cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte.

Reconstituted eggs were immediately fused after the injection of donor cells. The eggs were placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber overlaid with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 3 mg/ml of BSA. For the fusion, a single DC pulse of 150 V/mm was applied for 30 μsec using a BTX Electro Cell Manipulator 200 (BTX, San Diago, CA, USA). After fusion treatment, the eggs were maintained in TCM-199 supplemented with 3 mg/ml of BSA for 1 h and checked for fusion. The fused embryos were further activated 1 h after the fusion treatment by exposure to two DC pulses of 100 V/mm for 50 μsec each, followed by 4 h of culture with 2 mM 6-dimethlyaminopurine (6-DMAP) prior to *in vitro* culture.

***In Vitro* Culture of Embryos**

Following activation treatment, the SCNT embryos were cultured in 50 μl droplets of PZM-3 (Yoshioka *et al.* 2002) supplemented with 3 mg/ml of BSA overlaid with paraffin oil under an atmosphere of 5% CO_2 in humidified air at 39℃. In order to assess the inheritance of the donor centrosome, the fused embryos were fixed 15 min and 1 h after fusion. Nuclear progression and microtubule organization in the first mitotic phase of porcine SCNT embryos was evaluated at 20 h of fusion.

Immunofluorescent Staining and Confocal Microscopy

Microtubules and DNA were detected by an indirect immunocytochemical technique. Fused embryos and somatic cells were fixed with 3.7% (w/v) paraformaldehyde in PBS for 40 min and 15 min, respectively, at room temperature. Fixed embryos were maintained in PBS containing 3 mg/ml of BSA and 0.02% sodium azide for 1 week at 4℃. Fixed embryos and cells were permeabilized by transfer into PBS containing 0.1% (w/v) Triton X-100, 3 mg/ml of BSA and 0.02% sodium azide for 40 min and 10 min, respectively, at 39℃. After washing twice in PBS containing 0.01% Triton X-100 and 3 mg/ml of BSA, the samples were incubated in blocking solution (PBS containing 150 mM glycine, 3 mg/ml BSA, and 0.02% sodium azide) for 30 min and 5 min, respectively, at 39℃. In order to evaluate γ- and β-tubulins, double immunofluorescence labeling was conducted. The microtubules were immunolabeled with a mouse monoclonal antibody against β-tubulin (Sigma) and a rabbit monoclonal antibody against γ-tubulin (Sigma). Primary antibodies were detected using Alaxa-488 goat anti-mouse IgG and Alaxa-546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Antibodies and IgGs were diluted in blocking solution (1:200, w/v) prior to use. The samples were incubated with each primary and secondary antibody for 40 min at 39℃, respectively, followed by three washings for 5 min each. DNA was stained with 1 μg/ml of Hoechst 33342 for 20 min at 39℃. Finally, all samples were mounted on glass slides in Vecta-Shield anti-fade (Vector Laboratories, Burlingame, CA, USA) under a coverslip and were examined under a laser scanning confocal microscope (Zeiss LSM 510, Jena, Germany).

Statistical Analysis

Data were analyzed by Duncan's multiple-range tests using the General Linear Model procedure of the software package Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Spindle Microtubule Dynamics during the First Mitosis of Porcine Fetal Fibroblast Cells

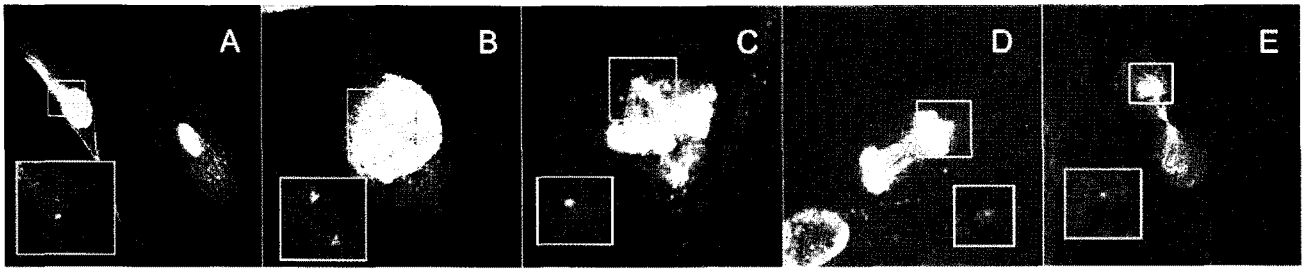

Fig. 1. Localization of γ-tubulin spot (centrosome) in various mitotic phases of porcine fetal fibroblast cells. (A) Interphase, (B) Prophase, (C) Metaphase, (D) Late anaphase (anaphase (B), (E) Cytokinesis. DNA (blue), β-tubulin (green) and γ-tubulin (red) are presented. A centrosome (inset) is shown in all of the mitotic stages.

One or two γ-tubulin spots (centrosome) were observed in all of the mitotic stages (Fig. 1). A centrosome was located near the nucleus at interphase (Fig. 1A), and spindle microtubules were organized from two separated spindle poles resulting from the duplication of the centrosome at prophase (Fig. 1B). Aligned chromosomes at the center of the mitotic spindle were moved toward the opposite two poles along with spindle elongation (Fig. 1C and D), after which cytokinesis was occurred (Fig. 1E).

Inheritance of the Donor Centrosome

One or two γ-tubulin spots were observed near the transferred nucleus 15 min after fusion in the majority of SCNT embryos (55/59, 93.2%, Fig. 2, and Fig. 3A and B). These spots are considered to be the inherited donor centrosome. The centrosome was not detected in the small proportion (4/59, 6.8%) 15 min after fusion (Fig. 2 and Fig. 3C), which is believed to be attributed to the abnormality of the transferred donor cell. One hour after fusion, the frequency of embryos manifesting γ-tubulin spots in SCNT embryos was 38.6% (22/57, Fig. 2). In the majority of SCNT embryos, neither γ-

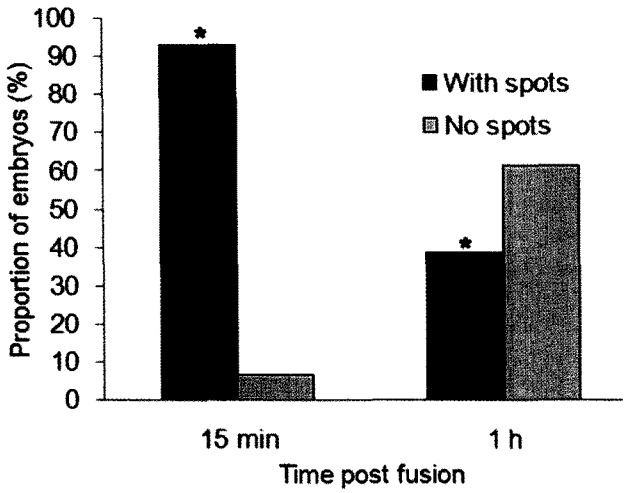

Fig. 2. Donor centrosome inheritance patterns in porcine SCNT embryos. γ-tubulin spots in porcine SCNT embryos was examined by laser scanning confocal microscopy 15 min and 1 h after fusion. The total numbers of embryos were 59 and 57 in 15 min and 1 h groups, respectively.

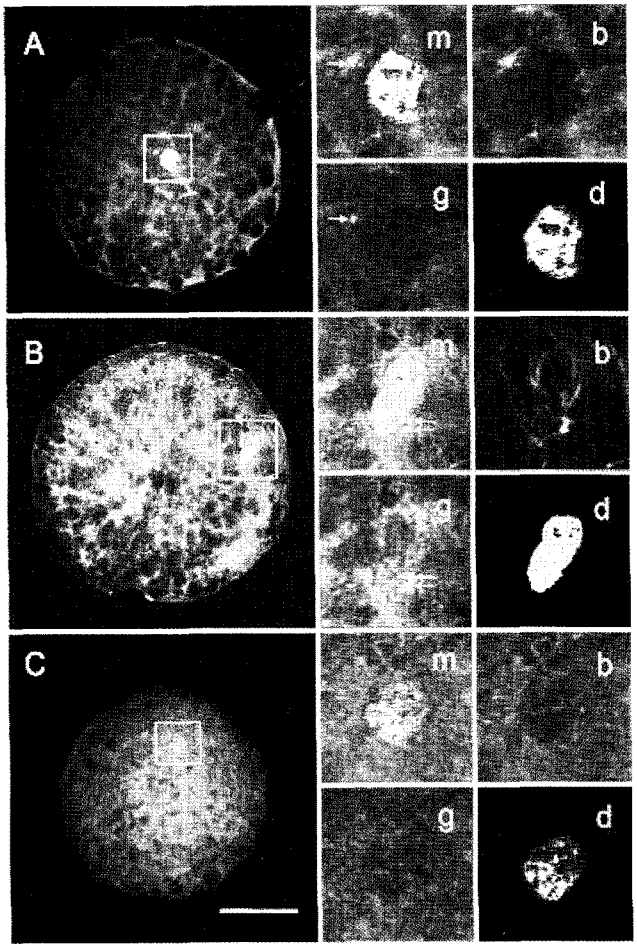

Fig. 3. Pattern of the centrosome inheritance and its localization in porcine SCNT embryos. Inheritance of the centrosome and its localization were assessed 15 min after fusion. (A) One- and (B) two-spots are shown near the transferred nucleus. (C) SCNT embryos do not exhibit an inherited centrosome. Insets indicate DNA (d; blue), β-tubulin (b; green), γ-tubulin (g; red) and merged (m) images. Scale bar indicates 50 μm.

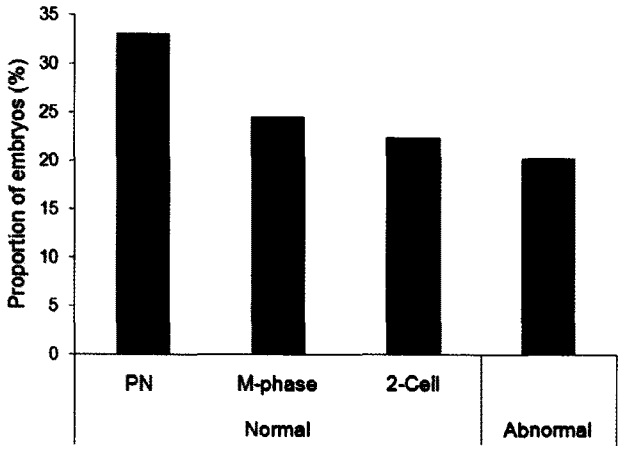

Fig. 4. Nuclear progression and microtubule organization in the first mitotic phase of porcine SCNT embryos. Nuclear progression and microtubule organization of SCNT embryos (n=94) were examined by confocal laser microscope 20 h after fusion. PN, pronucleus-structure; M-phase, the first mitotic phase; Abnormal, embryos belong to an abnormal category as represented at Fig. 6.

nor β-tubulins were observed.

Nuclear Progression and Microtubule Organization in the First Mitotic Phase of SCNT Embryos

Nuclear progression and microtubule organization in the first mitotic phase of porcine SCNT embryos were assessed 20 h after fusion (Fig. 4). A pronucleus (PN) structure was observed with a high frequency (31/94, 33.0%). The frequency of the M-phase and cleaved embryos were 24.5% (23/94) and 22.3% (21/94), respectively. The frequency of embryos evidencing an abnormal spindle (see below) was 20.2% (19/94).

Spindle Microtubule Dynamics during the First Mitosis of SCNT Embryos

The γ-tubulin was initially observed at the time of the occurrence of nuclear envelop break down (NEBD), and two γ-tubulin spots were noted around the periphery of the nucleus undergoing NEBD (Fig. 5A). After NEBD, β-tubulin (spindle) was organized from the γ-tubulin spots, and associated with the condensed chromosomes (Figs. 5B and C). Shortly after chromosome condensation, the metaphase chromosomes were surrounded by spindle microtubules, γ- and β-tubulins (Fig. 5D). During the ana-telophase transition, each condensed chromosome mass was translocated with two spindle poles and gradually disappeared, and the γ-tubulin spot as a centrosome was not observed (Fig. 5E).

Various abnormal mitosis configurations were observed in the porcine SCNT embryos (Fig. 6) including the organization of multiple spots (Fig. 6A and D), asymmetrical chromosomes separation by an acentrosomal spindle pole (Fig. 6B), the organization of two spots associated with misarranged chromosomes (Fig. 6C) and nucleus-unassociated spots (Fig. 6D and D'), and cytoplasmic fragmentation (Fig. 6E). The majority of the asymmetrical separation of chromosome and cytoplasmic fragments in SCNT embryos occurred as the result of the dysfunction of spindle microtubules owing to the abnormally organized centrosome (γ-tubulin spot).

DISCUSSION

In pigs, somatic cells harbor a centrosome in interphase, and it is likely that in the majority of mammalian somatic cells, the centrosome is duplicated and separated after the cell cycle progression. Although porcine mature oocytes harbor no centrosome (Kim *et al.*, 1996), parthenogenetically activated ones are cleaved normally and developed to the blastocyst stage, which can be explained by the existence of γ-tubulin in porcine oocytes. γ-tubulin is one of the materials comprising the pericentriolar region (Stearns *et al.* 1991) and can operate as the functional unit of MTOC (Raff *et al.*, 1993; Zheng *et al.*, 1995; Moudjou *et al.*, 1996). During meiotic maturation in mouse oocytes, many acentrosomal MTOCs are formed *de novo* associated with the nuclear envelope just prior to NEBD. These MTOCs are clustered and finally form the opposite two poles containing γ-tubulins (Schuh and Ellenberg, 2007).

By the SCNT procedure, the centrosome (γ-tubulin spot) is introduced into a recipient cytoplasm which is duplicated at S-phase-as is also the case in somatic cells-and located beside a PN-structure in bovine (Shin *et al.*, 2002). As in bovine SCNT embryos, the γ-tubulin spot which refer to as the donor centrosome and the β-tubulin of the donor cell were observed beside the transferred donor nucleus of porcine SCNT embryos immediately after fusion, however, these tubulins rapidly disappeared. Both tubulins in the porcine SCNT embryos were not detected 3 h after fusion, and did not appear until the first mitosis begins (data not shown), as reported previously (Zhong *et al.*, 2007). These results imply that the centrosome inherited from the donor cell dispersed gradually into the cytoplasm (Zhong *et al.*, 2005).

During the mitosis of somatic cells, DNA is duplicated in S-phase and condensed to a pair of chromosomes in M-phase. Along with the nuclear progressions, the centrosome is also duplicated and segregated during the prophase transition. In SCNT studies, the generation of several centrosomes around the periphery of the nucleus or spindle apparatus is believed to be one of the reasons for the occurrence of an abnormal chromosome separation (Sluder *et al.*, 1997; Dai

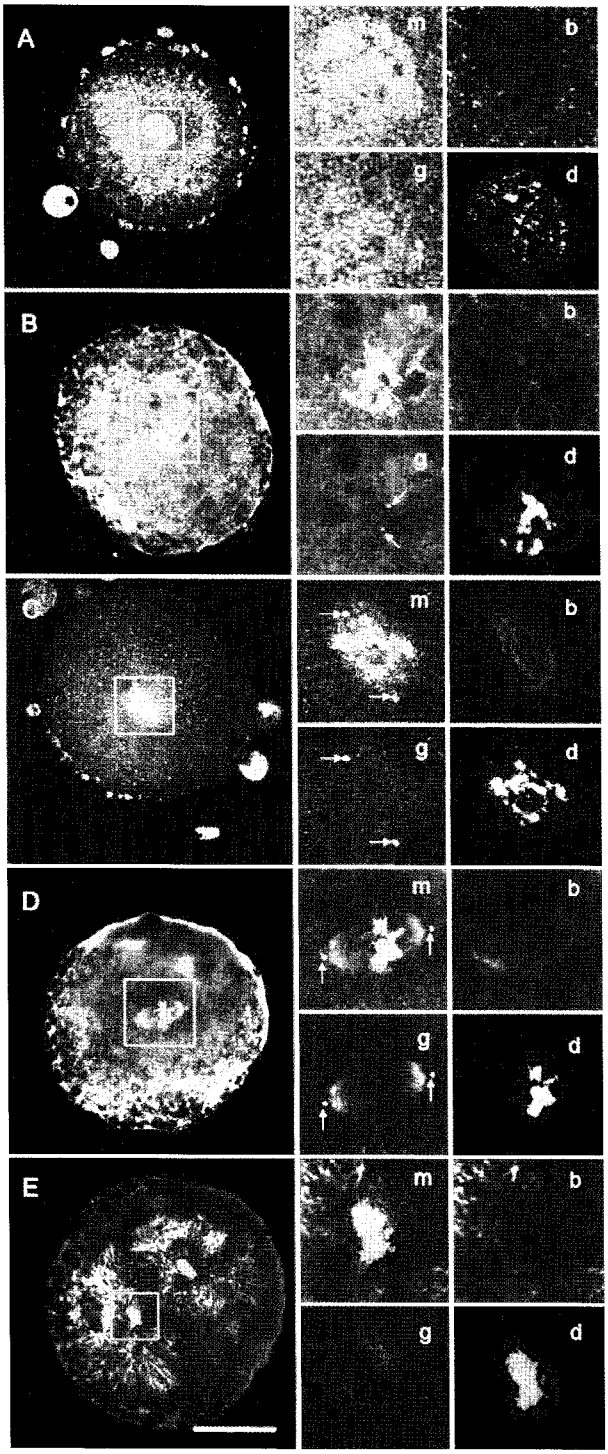

Fig. 5. Microtubule organization and localization in the first mitotic phase of porcine SCNT embryos. Nuclear progression and microtubule distribution in different phases of the first mitosis of SCNT embryos were evaluated 20 h after fusion. (A) Interphase; (B) Prophase; (C) Prometaphase; (D) Metaphase; (E) Ana-telophase, in which two separated chromosome masses were translocated with spindle poles (γ-tubulin dense region). Spots are detected only until the metaphase stage. Insets indicate DNA (d; blue), β-tubulin (b; green), γ-tubulin (g; red; arrows) and merged (m) images, respectively. Scale bar indicates 50 μm.

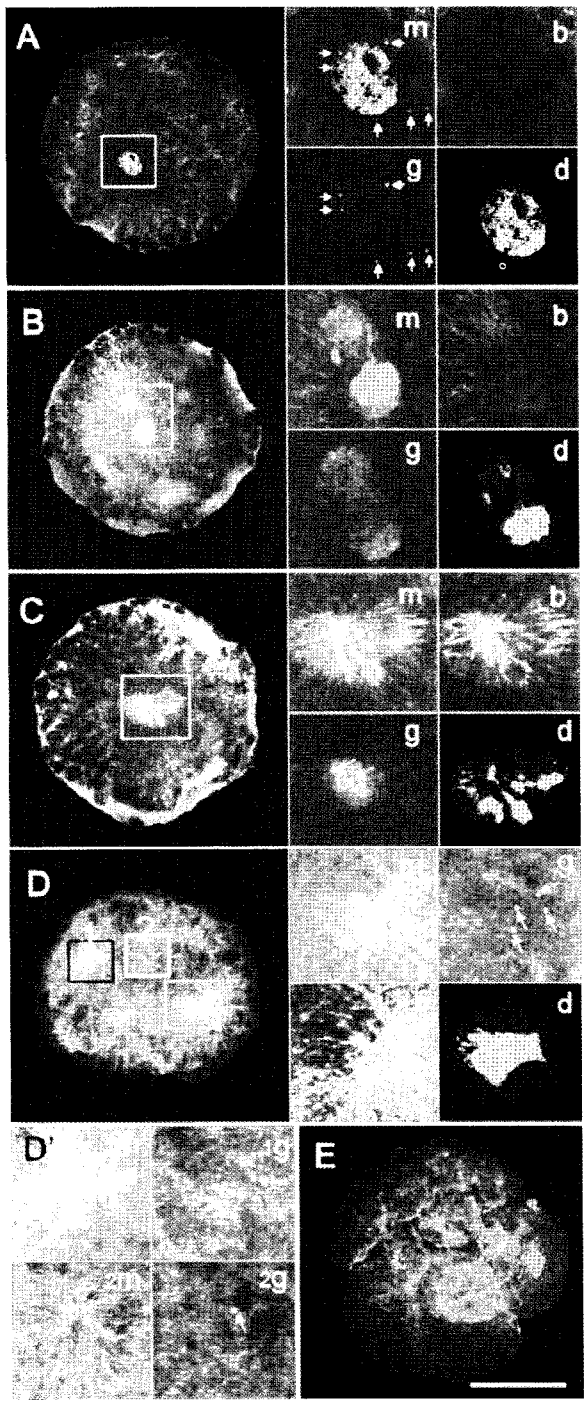

Fig. 6. Abnormal nuclear and microtubule configuration in the first mitotic phase of porcine SCNT embryos. (A) Multiple spots are seen around the nucleus, but some of the spots are not related with the nucleus. (B) No spot is observed in the SCNT embryo. (C) Two spots are observed around the condensed chromosomes, but the chromosomes are misarranged and the mitotic spindles are abnormally organized. (D) Spots are organized and distributed not only near the nucleus but also in the cytoplasm (1 and 2, and D'). (E) SCNT embryos underwent cytoplasmic fragmentation. Insets indicate DNA (d; blue), β-tubulin (b; green), γ-tubulin (g; red) and merged (m) images, respectively. Scale bar indicates 50 μm.

et al., 2006). Thusly, we confirmed that the majority of abnormal aspects in chromosome separation were induced by multiple centrosome numbers and dysfunction. Furthermore, the localization of the centrosome is also crucial to ensuring normal cleavage, because cytoplasmic fragmentation might occur due to the activity of multiple centrosomes organized independently from the nucleus.

Unlike the case in bovine SCNT embryos (Shin *et al.*, 2002; Dai *et al.*, 2006), the mitotic progression of a nucleus and microtubule dynamics (γ- and β-tubulins) in the first mitotic phase of porcine SCNT embryos was not similar to those of somatic cells. During first interphase, any microtubules associated with a pronucleus were not detected in the oocyte cytoplasm, but two γ-tubulin spots were observed around the periphery of the nucleus undergoing NEBD. We were unable to elucidate the origin of the γ-tubulin spot, but it is considered to be reorganized from inherited centrosomal materials. Two separated chromosome masses were translocated with spindle poles (γ-tubulin dense region) in the ana-telophase transition (see Fig. 5E), which is identical to the case of meiotic chromosome separation (Lee *et al.*, 2000), which can also occur when there is no centrosome in the spindle poles (Lee *et al.*, 2000). This finding indicates that the newly formed γ-tubulin spot (centrosome) may perform a certain function as a central MTOC, much as is the case in somatic cells. However, the processing of chromosome separation and microtubule dynamics after anaphase did not follow those of somatic cells, which may be considered evidence that the inherited centrosome has been newly reprogrammed.

In conclusion, we suggest that the donor centrosome is transferred into the recipient cytoplasm, but that it disappears or deteriorates in the cytoplasm until the resumption of the first mitosis, which is considered to be related to the reprogramming of the inherited centrosome. We also verified that the first mitosis of SCNT embryos is characterized by both the processing of spindle nucleation, as in somatic cells, and the progression of chromosome separation, as in meiotic oocyte division.

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